

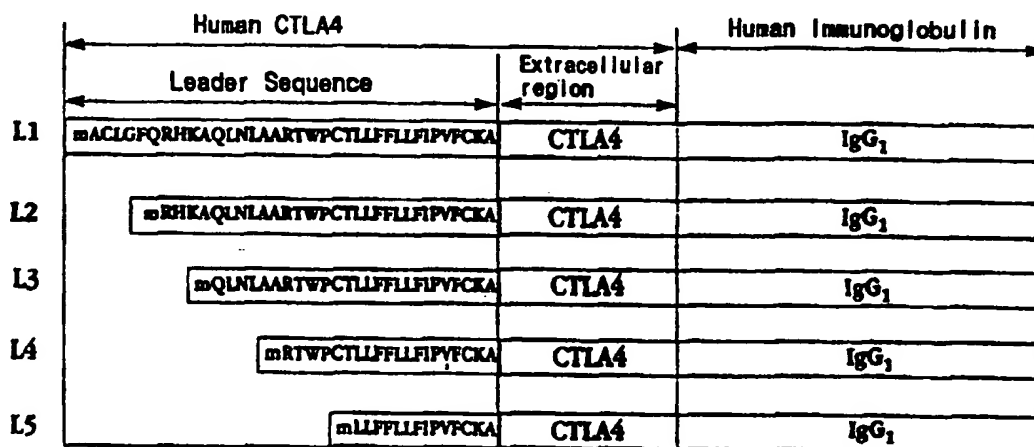
PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶ : C12N 15/62, A61K 38/00		A1	(11) International Publication Number: WO 98/31820
			(43) International Publication Date: 23 July 1998 (23.07.98)
(21) International Application Number: PCT/KR98/00009 (22) International Filing Date: 19 January 1998 (19.01.98) (30) Priority Data: 1997/1360 18 January 1997 (18.01.97) KR (71) Applicant (for all designated States except US): BORYUNG PHARMACEUTICAL CO., LTD. [KR/KR]; Boryung Building, 66-21, Wonnam-dong, Chongro-ku, Seoul 110-450 (KR). (71)(72) Applicant and Inventor: CHUNG, Yong-Hoon [KR/KR]; 405-804, Jukong Apt., Dunchon-dong, Kangdong-ku, Seoul 134-060 (KR). (74) Agent: SUH, Jong, Wan; New-Seoul Building, 3rd floor, 828-8, Yeoksam-dong, Kangnam-ku, Seoul 135-080 (KR).		(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, GW, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG). Published <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>	

(54) Title: A CTLA4-Ig FUSION PROTEIN HAVING HIGH TITER



(57) Abstract

The present invention relates to a CTLA4-Ig fusion protein, in which an extracellular region of the CTLA4 is connected to CH₂, CH₃, and CH₄ of IgM or to hinge, CH₂ and CH₃ of IgG1 Cys₃₀₈, and six monomers of which are polymerized to be a hexameric structure. According to the present invention, it is provided a CTLA4-Ig fusion protein having a decreased dosage and high titer.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece			TR	Turkey
BG	Bulgaria	HU	Hungary	ML	Mali	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MN	Mongolia	UA	Ukraine
BR	Brazil	IL	Israel	MR	Mauritania	UG	Uganda
BY	Belarus	IS	Iceland	MW	Malawi	US	United States of America
CA	Canada	IT	Italy	MX	Mexico	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NE	Niger	VN	Viet Nam
CG	Congo	KE	Kenya	NL	Netherlands	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NO	Norway	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	NZ	New Zealand		
CM	Cameroon			PL	Poland		
CN	China	KR	Republic of Korea	PT	Portugal		
CU	Cuba	KZ	Kazakhstan	RO	Romania		
CZ	Czech Republic	LC	Saint Lucia	RU	Russian Federation		
DE	Germany	LI	Liechtenstein	SD	Sudan		
DK	Denmark	LK	Sri Lanka	SE	Sweden		
EE	Estonia	LR	Liberia	SG	Singapore		

A CTLA-4 Ig FUSION PROTEIN HAVING HIGH TITER

Technical Field

The present invention relates to a CTLA4-Ig fusion protein
5 having high titer, and more particularly, to a fusion protein connecting an
extracellular region of CTLA4 and C μ of IgM or C γ 1 region of IgG.

Background Art

10 In organ transplant, fatal to a patient is the rejection by
immunoreaction which occurs by discriminating self and non-self.

In the rejection of the organ transplant, T-cell plays an important
role. The reaction of T-cell starts with two kinds of signals, an antigen-
sensitive stimulatory and a costimulatory signals. A large number of
ligand/receptor bonds including ICAM-1/LFA-1, B7/CD28 and CTLA4
15 and LFA-3/CD2 participate in the costimulatoion. Especially, CD28 plays
an important role in the reaction of the T-cell, making stable mRNA of a
T-cell cytokinin by binding to the B7.1 and B7.2(June, C. H. *et al.*, *Mol.*
Cell Biol., 7, 4472, 1987/Lindstent, *et al.*, *Science*, 244, 339, 1989), and
increasing the productivity of interleukin-2(IL-2), interferon- γ (IFN- γ),
20 tumor necrosis factor- α (TNF- α), lymphotoxin, granulocyte
macrophage-colony stimulating factor(GM-CSF), and interleukin-3(IL-
3).

Thus if the costimulation by the CD28 is blocked by inhibiting
binding of the CD28 and the B7.1 and B7.2, the rejection of the organ
25 transplant can be suppressed.

CTLA4 has 67% homology with the CD28, binding to the B7(B7.1
and B7.2) of an antigen presenting cell(APC) like CD28. Linsley *et al.*
reported that a monomeric CTLA4-Ig fusion protein was prepared by
fusing the CTLA4 and an IgG, and that the protein has the
30 immunosuppression effect (Linsley, P. S. *et al.*, *J. Exp. Med.* 174, 561,
1991). Yamada *et al.* recently reported that they manufactured a

pentameric CTLA4-IgM fusion protein and that the protein extended lives of patients after the organ transplant (Yamada, A. *et al.*, *Microbio. Immunol.*, **40**, 513~518, 1996)

However, the CTLA4-Ig fusion protein, since its too much dosage of 600 mg per once for a 60 kg adult and high manufacturing cost, is hardly commercially viable.

Disclosure of the Invention

According to one aspect of the present invention, there is provided a CTLA4-Ig fusion protein in which an extracellular region is connected with CH₂, CH₃, and CH₄ region of IgM, or with a hinge, CH₂, and CH₃ of IgG1 Cys₃₀₈(IgG1 having Cys₃₀₈), and which has a hexameric structure.

The hexameric structure of the CTLA4-Ig fusion protein is caused by forming multimer between adjoining IgMs or between IgG1 Cys₃₀₈s forced by disulfide bonds of cysteins. To put it concretely, Cys₄₁₄ and Cys₅₆₇ of the IgM make a disulfide bond and, in case of IgG1 Cys₃₀₈s of IgG1s make a disulfide bond. The IgG1 Cys₃₀₈ of the present invention is the one that Leu₃₀₈ of the IgG1 CH₂ region, the correspondent site of Cys₄₁₄ of IgM, is converted to cystein in order to form polymeric IgG1 like IgM.

According to another aspect of the present invention, there is provided DNA base sequence coding amino acid sequence correspondent to the CTLA4-Ig fusion protein.

According to still another aspect of the present invention, there is provided the expression vectors pHIGH3neo and pHIGHgpt manufactured by inserting to vectors of pSV2neo and pSV2gpt an enhancer, a promoter, CTLA4 leader sequence of which N-terminal is cut, and DNA sequence coding amino acid sequence correspondent to the CTLA4-Ig fusion protein. The CTLA4 leader sequence of which N-terminal is cut makes the CTLA-Ig fusion protein secreted to the outside of cell.

According to still another aspect to the present invention, there is provided a transformed body manufactured by inserting to a mouse SP2/0-Ag14 cell the expression vectors pHIGH3neo and pHIGH3gpt which is manufactured by inserting to the vectors pSV2neo and pSV2gpt an enhancer, a promoter, CTAL4 leader sequence of which N-terminal is cut, and the DNA sequence coding amino acid sequence correspondent to the CTLA4-Ig fusion protein.

According to still another aspect of the present invention, there is provided an immunosuppressant containing the CTLA4-Ig fusion protein. The CTLA4-Ig fusion protein of the present invention, a soluble protein, binds to the B7 of the antigen presenting cell to inhibit binding of the CTLA4 and the CD28 of T-cell at the B7, to block costimulatory signal needed for the activation of T-cell and, in the result, the immunoreaction is suppressed.

By the features of the present invention, the titer of the CTLA4-Ig fusion protein according to the present invention is 32~356 times of an existing CTLA4-Ig fusion protein. The dosage of the CTLA4-Ig fusion protein according to the present invention is 2~13 mg per once for a 60 kg adult, and it's effective titer is 45~260 times of the existing CTLA4-Ig fusion protein's.

Brief Description of the Drawings

The above objects, and other features and advantages of the present invention will become more apparent after a reading of the following detailed description taken in conjunction with the drawings, in witch:

Fig.1 is a structure of a CTLA4 gene cloned by a reverse transcription-polymerase chain reaction(RT-PCR) of example 1.

Fig.2 is a expression ratio of a fusion protein of example 2.

Fig. 3a, 3b are base sequences of a CTLA4-IgM fusion gene of example 2 and an correspondent amino acid sequence thereof.

Fig. 4a, 4b are base sequence of a CTLA4-IgG1 Cys₃₀₈ fusion gene of example 3 and a correspondent amino acid sequence thereof.

Fig. 5a, 5b are a manufacturing method for the expression vectors of pHIGH3neo and pHIGH3gpt of the CTLA4-IgM fusion gene and the CTLA4-IgG1 Cys₃₀₈ fusion gene.

Fig. 6a, 6b are western blots of the CTLA4-IgM fusion protein and the CTLA4-IgG1 Cys₃₀₈ fusion protein.

Fig. 7 is a structure of 600kD of the CTLA4-IgM fusion protein or the CTLA4-IgG Cys₃₀₈ fusion protein.

Fig. 8 is a graph showing the immunosuppression effect of the CTLA4-IgM fusion protein and the CTLA4-IgG1 Cys₃₀₈ fusion protein.

Best Mode for Carrying out the Invention

The present invention is further illustrated in the following example, which should not be taken to limit the scope of the invention.

Example 1: Cloning of human CTLA4, IgG1, and IgM genes

CTLA4, IgG1, and IgM genes were cloned respectively by the method of a reverse transcription-polymerase chain reaction(RT-PCR).

1. Cloning of the CTLA4 gene

A template used for the cloning of the CTLA4 gene by the reverse transcription-polymerase chain reaction was mononucleocyte mRNA of a healthy adult. The mRNA was separated as follows:

Blood taken from a healthy adult was density-gradient centrifuged using Ficoll-Hypaque to obtain monocyte cell layer. By adding RPMI-1640 medium containing 10% bovine fetus to the above monocytes 5X10⁵ monocytes/ml was made and here leukoagglutinin(Pharmacia Corp.) added to be 3.5 μ g/ml. The mixture was incubated 36~48 hours under the condition of 5% CO₂, 37°C in order to separate mRNA.

The polymerase used in the reverse transcription-polymerase chain reaction was pfu(Stratagene Corp.).

The primers used in the reverse transcription-polymerase chain

reaction are five forward primers(L1~5) and a reverseward primer, as follows;

Forward primers

L1 5'-ATG GCT TGC CTT GGA TTT CAG-3'

L2 5'-ATG CGG CAC AAG GCT CAG CTG AAC-3'

L3 5'-ATG CAG CTG AAC CTG GCT GCC AGG-3'

L4 5'-ATG AGG ACC TGG CCC TGC ACT CTC-3'

L5 5'-ATG CTC CTG TTT TTT CTT CTC TTC-3'

Reverseward primer

5'-CTC TGC AGA ATC TGG GCA CGG TTC AGG ATC-3'

It is invented for the L1 primer to be expressed as an original CTLA4 without cutting, for the L2 primer as a form that 6 amino acids of it were cut from N-terminal, 11 amino acids cut for the L3, 16 amino acids cut for the L4, and 22 amino acids cut for the L5 from N-terminal (Fig.1).

Inventing the forward primers to be expressed as cutting form of amino acids from N-terminal is for a part of leader sequence to be cut and expressed, and for the CTLA4 protein to be secreted to an extracellular region. And 5 primers were invented in order that the leader sequence is cut and expressed one by one for the determination of a leader sequence which makes the most CTLA4 proteins secreted to extracellular region.

CTLA4 gene obtained by the reverse transcription-polymerase reaction was cloned to pUC 18. The cloned CTLA4 gene has confirmed which base No.49 was converted from adenine to guanine, and base No.331 was converted from guanine to adenine. In the result, an amino acid No.17 of CTLA4 protein was converted from threonine to alanine, and an amino acid No.111 of CTLA4 protein was converted from alanine to threonine.

2. Cloning of IgG1 gene

The cloning method was same with the method of the above 1 of the example 1 except template and primer. The template used here was mRNA of B-cell at peripheral blood lymph node obtained from a recovering ill-defined fever patient. The primer was invented in order to clone a counterbalancing of IgG1 as follows;

Forward primer

5'-A TCT GCA GAG CCC AAA TCT TGT GAC-3'

Reverseward primer

5'-TT CTC GAG TCA TTT ACC CGG AGA CAG GGA-3'

3. Cloning of IgM gene

Same with the method of the above 2 of the example 1 except primer. The primer was invented in order to clone a counterbalancing of the IgM as follows;

Forward primer

5'-GAC TGC AGA GCT GCC TCC CAA AGT G-3'

Reverseward primer

5'-GTA GCA GGT GCC AGC TGT GTC TGA-3'

Example 2: Determination of the optimum leader sequence for extracellular secretion

The five CTLA4 genes obtained by serial deletion of N-terminal amino acids were fused with IgG1 respectively, inserted to a vector pHIGH3, and transfected to a mouse bone marrow SP2/0-Ag14 cell(ATCC#: CRL 1581) to be expressed. And after an incubation for 48 hours, the expression ratio was analyzed by a cell circulation assay.

The result of the above analysis shows that in case of L1 primer 4.9% of the fusion protein, 3.1% for L2 primer, 0% for L3 primer, 7.8% for L4 primer and 6% for L5 primer are expressed (Fig.2). It confirms that the leader sequence deleted of 16 amino acids from N-terminal, obtained by using L-4 primer, makes the most fusion proteins secreted

most to an extracellular region.

Example 3: Manufacturing of IgG1 Cys₃₀₈

5 IgG1 Cys₃₀₈ was manufactured by converting Leu₃₀₈ of IgG1 to cysteine using a polymerase chain reaction. The primers used in the polymerase chain reaction are as follows;

Forward primer

5'-A TCT GCA GAG CCC AAA TCT TGT GAC-3'

10 Reverseward primer

5'-TT CTC GAG TCA TTT ACC CGG AGA CAG GGA-3'

Converting primer

5'-CCA GTC CTG GTG ACA GAC GGT GAG GAC-3'

15 First, the primary polymerase chain reaction using the forward primer and reverseward primer was performed, and then using the product of the above reaction and reverseward primer, secondary polymerase chain reaction was performed. The amplified product of the secondary polymerase chain reaction was cloned in pUC18 vector.

Example 4: Construction of the expression vector of the CTLA4-Ig fusion gene

20 Genome DNA of SP2/0-Ag14 cell was extracted, cut with restriction enzymes of BamH I and Hind III, transferred to a nitrocellulose membrane, and performed Southern blot with 5'-ATT TGC ATA TTT GCA TAT TTG CAT-3' fragment and 5'-CTC ATG ACT CAT GAC TCA-3' fragment marked with isotope to clone 5.3kb promoter.

30 On the other hand genome DNA of SP2/0-Ag14 cell was cut by restriction enzymes of EcoR I and BamH I and performed the southern blot with 5'-TGA ATT GAG CAA TGT TGA ATT GAG CAA TGT-3' fragment and 5'-TAT TTG GGG AAG GGT ATT TGG GGA AGG-3' fragment marked with isotope to clone 1kb enhancer.

An enhancer-promoter-CTLA4-

Ig fusion gene was cloned to pUC 18 by fusing the 1kb enhancer and 5.3 kb promoter in pUC 19, and inserting the fused product to the site of Sal I, the front part of CTLA4-

Ig fusion gene cloned in pUC 18(CTLA4-

IgM fusion gene of the example 2 and CTLA4-

IgG1 Cys₃₀₈ fusion gene of the example 3, Fig.3a,3b and Fig.4a, 4b). By cutting only enhancer-promoter-CTLA4-

Ig fusion gene by treating EcoR I and Hind III to the above clone

and then by inserting the clone to pSV2neo and pSV2gpt, the expression vectors of pHIGH3neo and pHIGH3gpt was constructed (Fig . 5a, 5b).

Example 5: Expression of CTLA4-Ig fusion gene and purification of CTLA4-Ig fusion protein

SP2/0-Ag14 cell of mouse was incubated in 10% FCS-DMEM medium, and diluted to 5×10^6 cells/ml by adding PBS. The above suspension 0.2ml was put to cuvette(BioRad Corp.) for electroporation and the purified expression vector 15 μ g of the CTLA4-Ig fusion gene of example 4 was added. And then electroporation (BT \times 820) was performed under the condition of 480V, 99 μ sec, 2cycle.

The above cells were incubated for 3 weeks in the FCS-DMEM medium containing 1500 μ g/ml of geneticin G418(Gibco Corp.). And then colonies were separated, collected, and incubated for amplifying. The CTLA4-Ig fusion gene expression was examined by the a cell circulation analyzer and enzyme linked immunosorbent assay(ELISA) method.

These cells were incubated in large quantities in FCS-medium and the CTLA4-Ig fusion protein was precipitated by ammonium sulfate addition. And then by an affinity chromatography using protein A, the CTLA4-Ig fusion protein was purified.

In order to fine out the biochemical properties of the CTLA4-Ig fusion protein, electrophoresis and western blot were performed(F

ig. 6a, 6b). The result shows that there are two kinds of the CTL A4-IgM fusion protein and six kinds of the CTLA4-IgG1 fusion protein. Among these, CTLA4-Ig fusion protein of 600kD was separated and purified. The CTLA4-Ig fusion protein of 600kD is 6 times as large as the existing CTL A4-Ig fusion protein(100kD), and is a hexamer which was six of CTL A4-Ig fusion protein polymerized (Fig.7).

Example 6: Immunosuppression effect of the CTLA4-Ig fusion protein

The existing CTLA4-Ig fusion protein is a comparative example 1, the pentameric CTLA4-Ig fusion protein is a comparative example 2, and the hexameric CTLA4-Ig fusion protein is an example. and the Immunosuppression effects of them were examined as follows;

From two healthy adults peripheral blood lymphocytes were separated, and on the cells of the one person 300 rad of ^{60}Co radiation was irradiated.

The cells of the two persons were spread into a 96-well plate with 2.5×10^4 cells/ml, respectively. And after incubating for 88~96 hours under the condition of 37°C , 5% CO_2 , added $0.5 \mu\text{Ci}$ ^3H -thymidine(NEN Research product) per well and incubated 5 hours again.

The incubated cells were adsorbed to a glass filter by using titertek(Flow lab), put into a test tube, and after adding $5\mu\text{l}$ of Scintillation cocktail a radioactivity was measured by using β -liquid scintillation counter. The all tests were performed three for every times under the same condition and an average of them was determined. The percent value gained by adding the fusion protein of the present invention was calculated on the basis of the radiation value(100%) gained without an addition. And when the value reaches to 50%, the value was defined as a line of 50% division suppression and the titer between fusion proteins was compared on the basis of the concentration of the adding

fusion protein.

As a result, the 50% division suppression concentration of the CTLA4-Ig fusion protein of this example is 0.009~0.022 $\mu\text{g}/\text{ml}$ (the average is 0.016 $\mu\text{g}/\text{ml}$). This value is lower than 0.7~3.2 $\mu\text{g}/\text{ml}$ (the average is 1.4 $\mu\text{g}/\text{ml}$) of the comparative example 1 and lower than 0.031~0.056 $\mu\text{g}/\text{ml}$ (the average is 0.44 $\mu\text{g}/\text{ml}$) the comparative example 2 (Fig.8). CTLA4-Ig fusion protein of this example has high titer, 32~356 times (the average is 88 times) comparing to the existing CTLA4-Ig fusion protein of the comparative example 1 .

CLAIMS

1. A CTLA4-IgM fusion protein, wherein an extracellular region of a CTLA4 is connected with CH₂, CH₃, and CH₄ of IgM, and which has a hexameric structure by polymerization of 6 monomers thereof.

2. A DNA sequence of Fig.4a, 4b coding the amino acid sequence corresponding to the CTLA4-IgM fusion protein as claimed in Claim 1.

3. A expression vector pHIGH3neo which is constructed by connecting an enhancer, a promoter and a CTLA4 of which N-terminal is cut, and DNA sequence coding amino acid sequence correspondent to the CTLA4-IgM fusion protein of Claim 1, and then by inserting the DNA sequence into vector pSV2neo.

4. A expression vector pHIGH3neo as claimed in Claim 3, wherein 16 amino acids of a leader sequence are cut from N-terminal.

5. A transformed body manufactured by inserting to a mouse SP2/0-Ag14 the expression vector pHIGH3neo constructed by connecting an enhancer, a promoter and a CTLA4 of which N-terminal is cut, and DNA sequences coding amino acid sequence correspondent to the CTLA4-IgM fusion protein of Claim 1, and then by inserting them into vector pSV2neo.

6. An immunosuppression medicine containing the CTLA4-IgM fusion protein in Claim 1.

7. A CTLA4-IgG1 Cys₃₀₈ fusion protein, wherein the extracellular region of CTLA4 is connected with a hinge, CH₂ and CH₃ of the IgG1 Cys₃₀₈ (IgG1 having Cys₃₀₈) and which has a hexameric structure by polymerization of 6 fusion protein monomers thereof.

8. A DNA sequence of Fig.3a, 3b coding amino acid sequence correspondent to the CTLA4-IgG1 Cys₃₀₈ fusion protein in Claim 7.

9. An expression vector pHIGH3neo which is constructed by connecting an enhancer, a promoter, and CTLA4 of which N-terminal is cut, and DNA sequence coding amino acid sequences correspondent to the CTLA4-IgG1 Cys₃₀₈ fusion protein in Claim 7, and then by inserting

them into a vector pSV2neo.

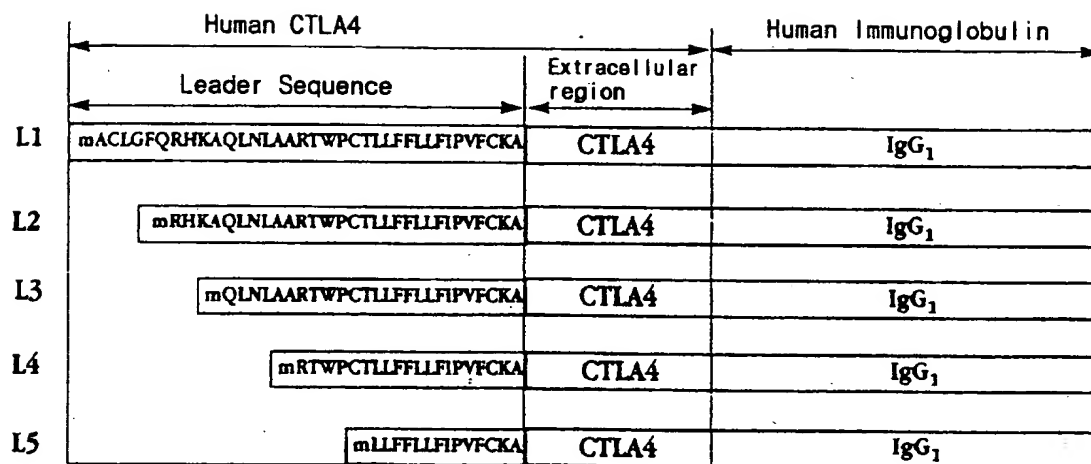
10. An expression vector pHIGH3neo as claimed in Claim 9, which 16 amino acids of a leader sequence are cut from N-terminal.

11. A transformed body which is manufactured by inserting to a
5 mouse SP2/0-Ag14 cell the expression vector pHIGH3neo constructed by connecting an enhancer, a promoter, and CTLA4 of which N-terminal is cut, and DNA sequence coding amino acid sequences correspondent to the CTLA4-IgG1 Cys₃₀₈ fusion protein in Claim 7, and then by inserting them into a vector pSV2neo.

10 12. An immunosuppressant containing the CTLA4-IgG1 Cys₃₀₈ fusion protein in Claim 7.

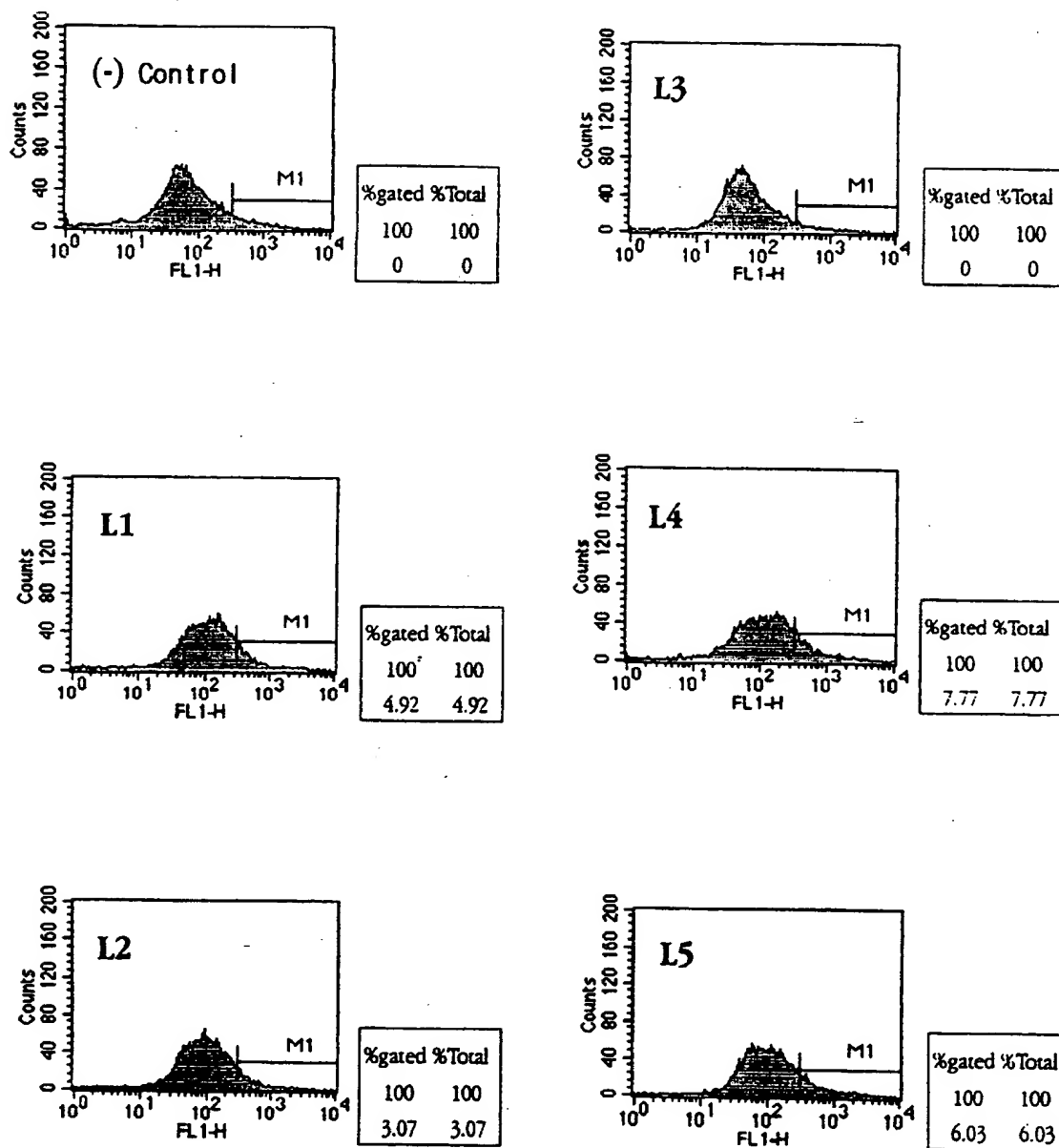
1/12

Fig. 1



2/12

Fig. 2



3/12

Fig. 3a

A	M	H	V	A	Q	P	A	V	V
GCA	ATG	CAC	GTG	GCC	CAG	CCT	GCT	GTG	GTA
L	A	S	S	R	G	I	A	S	F
CTG	GCC	AGC	AGC	CGA	GGC	ATC	GCC	AGC	TTT
V	C	E	Y	A	S	P	G	K	A
GTG	TGT	GAG	TAT	GCA	TCT	CCA	GGC	AAA	GCC
T	E	V	R	V	T	V	L	R	Q
ACT	GAG	GTC	CGG	GTG	ACA	GTG	CTT	CGG	CAG
A	D	S	Q	V	T	E	V	C	A
GCT	GAC	AGC	CAG	GTG	ACT	GAA	GTC	TGT	GCG
A	T	Y	M	M	G	N	E	L	T
GCA	ACC	TAC	ATG	ATG	GGG	AAT	GAG	TTG	ACC
F	L	D	D	S	I	C	T	G	T
TTC	CTA	GAT	GAT	TCC	ATC	TGC	ACG	GGC	ACC
S	S	G	N	Q	V	N	L	T	I
TCC	AGT	GGA	AAT	CAA	GTG	AAC	CTC	ACT	ATC
Q	G	L	R	A	M	D	T	G	L
CAA	GGA	CTG	AGG	GCC	ATG	GAC	ACG	GGA	CTC
Y	I	C	K	V	E	L	M	Y	P
TAC	ATC	TGC	AAG	GTG	GAG	CTC	ATG	TAC	CCA
P	P	Y	Y	L	G	I	G	N	G
CCG	CCA	TAC	TAC	CTG	GGC	ATA	GGC	AAC	GGA
T	Q	I	Y	V	I	D	P	E	P
ACC	CAG	ATT	TAT	GTA	ATT	GAT	CCA	GAA	CCG
C	P	D	S	A	E	P	K	S	C
TGC	CCA	GAT	TCT	GCA	GAG	CCC	AAA	TCT	TGT
D	K	T	H	T	C	P	P	C	P
GAC	AAA	ACT	CAC	ACA	TGC	CCA	CCG	TGC	CCA
A	P	E	L	L	G	G	P	S	V
GCA	CCT	GAA	CTC	CTG	GGG	GGA	CCG	TCA	GTC
F	L	F	P	P	K	P	K	D	T
TTC	CTC	TTC	CCC	CCA	AAA	CCC	AAG	GAC	ACC
L	M	I	S	R	T	P	E	V	T
CTC	ATG	ATC	TCC	CGG	ACC	CCT	GAG	GTC	ACA

Extracellular
region

CTLA-4

Hinge
region

4/12

Fig. 3b

C	V	V	V	D	V	S	H	E	D	
TGC	GTG	GTG	GTG	GAC	GTG	AGC	CAC	GAA	GAC	
P	E	V	K	F	N	W	Y	V	D	
CCT	GAG	GTC	AAG	TTC	AAC	TGG	TAC	GTG	GAC	
G	V	E	V	H	N	A	K	T	K	
GGC	GTG	GAG	GTG	CAT	AAT	GCC	AAG	ACA	AAG	
P	R	E	E	Q	Y	N	S	T	Y	
CCG	CGG	GAG	GAG	CAG	TAC	AAC	AGC	ACG	TAC	
R	V	V	S	V	L	T	V	C	H	
CGG	GTG	GTC	AGC	GTC	CTC	ACC	GTC	TGT	CAC	
Q	D	W	L	N	G	K	E	Y	K	
CAG	GAC	TGG	CTG	AAT	GGC	AAG	GAG	TAC	AAG	
C	K	V	S	N	K	A	L	P	A	
TGC	AAG	GTC	TCC	AAC	AAA	GCC	CTC	CCA	GCC	
P	I	E	K	T	I	S	K	A	K	
CCC	ATC	GAG	AAA	ACC	ATC	TCC	AAA	GCC	AAA	
G	Q	P	R	E	P	Q	V	Y	T	
GGG	CAG	CCC	CGA	GAA	CCA	CAG	GTG	TAC	ACC	
L	P	P	S	R	D	E	L	T	K	
CTG	CCC	CCA	TCC	CGG	GAT	GAG	CTG	ACC	AAG	
N	Q	V	S	L	T	C	L	V	K	
AAC	CAG	GTC	AGC	CTG	ACC	TGC	CTG	GTC	AAA	
G	F	Y	P	S	D	I	A	V	E	
GGC	TTC	TAT	CCC	AGC	GAC	ATC	GCC	GTG	GAG	
W	E	S	N	G	Q	P	E	N	N	
TGG	GAG	AGC	AAT	GGG	CAG	CCG	GAG	AAC	AAC	
Y	K	T	T	P	P	V	L	D	S	
TAC	AAG	ACC	ACG	CCT	CCC	GTG	CTG	GAC	TCC	
D	G	S	S	F	L	Y	S	K	L	
GAC	GGC	TCC	TCC	TTC	CTC	TAC	AGC	AAG	CTC	
T	V	D	K	S	R	W	Q	Q	G	
ACC	GTG	GAC	AAG	AGC	AGG	TGG	CAG	CAG	GGG	
N	V	F	S	C	S	V	M	H	E	
AAC	GTC	TTC	TCA	TGC	TCC	GTG	ATG	CAT	GAG	
A	L	H	N	H	Y	T	Q	K	S	
GCT	CTG	CAC	AAC	CAC	TAC	ACG	CAG	AAG	AGC	
L	S	L	S	P	G	K	.			
CTC	TCC	CTG	TCT	CCG	GGT	AAA	TGA			

CH₂ regionIgG₁-Cys₃₀₈CH₃ region

5/12

Fig. 4a

A	M	H	V	A	Q	P	A	V	V		
GCA	ATG	CAC	GTG	GCC	CAG	CCT	GCT	GTG	GTA		
L	A	S	S	R	G	I	A	S	F		
CTG	GCC	AGC	AGC	CGA	GGC	ATC	GCC	AGC	TTT		
V	C	E	Y	A	S	P	G	K	A		
GTG	TGT	GAG	TAT	GCA	TCT	CCA	GGC	AAA	GCC		
T	E	V	R	V	T	V	L	R	O		
ACT	GAG	GTC	CGG	GTG	ACA	GTG	CTT	CGG	CAG		
A	D	S	Q	V	T	E	V	C	A		
GCT	GAC	AGC	CAG	GTG	ACT	GAA	GTC	TGT	GCG		
A	T	Y	M	M	G	N	E	L	T		
GCA	ACC	TAC	ATG	ATG	GGG	AAT	GAG	TTG	ACC		
F	L	D	D	S	I	C	T	G	T		
TTC	CTA	GAT	GAT	TCC	ATC	TGC	ACG	GGC	ACC		
S	S	G	N	Q	V	L	T	I	I		
TCC	AGT	GGA	AAT	CAA	GTG	AAC	CTC	ACT	ATC		
Q	G	L	R	A	M	D	T	G	L		
CAA	GGA	CTG	AGG	GCC	ATG	GAC	ACG	GGA	CTC		
Y	I	C	K	V	E	L	M	Y	P		
TAC	ATC	TGC	AAG	GTG	GAG	CTC	ATG	TAC	CCA		
P	P	Y	Y	L	G	I	G	N	G		
CCG	CCA	TAC	TAC	CTG	GGC	ATA	GGC	AAC	GGA		
T	Q	I	Y	V	I	D	P	E	P		
ACC	CAG	ATT	TAT	GTA	ATT	GAT	CCA	GAA	CCG		
C	P	D	S	A	E	L	P	K	K		
TGC	CCA	GAT	TCT	GCA	GAG	CTG	CCT	CCC	AAA		
V	S	V	F	V	P	P	R	D	G		
GTG	AGC	GTC	TTC	GTC	CCA	CCC	CGC	GAC	GGC		
F	F	G	N	P	R	K	S	K	L		
TTC	TTC	GGC	AAC	CCC	CGC	AAG	TCC	AAG	CTC		
I	C	Q	A	T	G	F	S	P	R		
ATC	TGC	CAG	GCC	ACG	GGT	TTC	AGT	CCC	CGG		
Q	I	Q	V	S	W	L	R	E	G		
CAG	ATT	CAG	GTG	TCC	TGG	CTG	CGC	GAG	GGG		
K	Q	V	G	S	G	V	T	T	D		
AAG	CAG	GTG	GGG	TCT	GGC	GTC	ACC	ACG	GAC		
Q	V	Q	A	E	A	K	E	S	G		
CAG	GTG	CAG	GCT	GAG	GCC	AAA	GAG	TCT	GGG		
P	T	T	Y	K	V	T	S	T	L		
CCC	ACG	ACC	TAC	AAG	GTG	ACC	AGC	ACA	CTG		
T	I	K	E	S	D	W	L	G	Q		
ACC	ATC	AAA	GAG	AGC	GAC	TGG	CTC	GGC	CAG		
S	M	F	T	C	R	V	D	H	R		
AGC	ATG	TTC	ACC	TGC	CGC	GTG	GAT	CAC	AGG		
G	L	T	F	Q	Q	N	A	S	S		
GGC	CTG	ACC	TTC	CAG	CAG	AAT	CGC	TCC	TCC		
M	C	V	P	D	Q	D	T	A	I		
ATG	TGT	GTC	CCC	GAT	CAA	GAC	ACA	GCC	ATC		
R	V	F	A	I	P	P	S	F	A		
CGG	GTC	TTC	GCC	ATC	CCC	CCA	TCC	TTT	GCC		

Extracellular
region

CTLA-4

CH₂ region

6/12

Fig. 4b

S	I	F	L	T	K	S	T	K	L		
AGC	ATC	TTC	CTC	ACC	AAG	TCC	ACC	AAG	TTG		
T	C	L	V	T	D	L	T	T	Y		
ACC	TGC	CTG	GTC	ACA	GAC	CTG	ACC	ACC	TAT		
D	S	V	T	I	S	W	T	R	Q		
GAC	AGC	GTG	ACC	ATC	TCC	TGG	ACC	CGC	CAG		
N	G	E	A	V	K	T	H	T	N		
AAT	GGC	GAA	GCT	GTG	AAA	ACC	CAC	ACC	AAC		
I	S	E	S	H	P	N	A	T	F		
ATC	TCC	GAG	AGC	CAC	CCC	AAT	GCC	ACT	TTC		
S	A	V	G	E	A	S	I	C	E		
AGC	GCC	GTG	GGT	GAG	GCC	AGC	ATC	TGC	GAG		
D	D	W	N	S	G	E	R	F	T		
GAT	GAC	TGG	AAT	TCC	GGG	GAG	AGG	TTC	ACG		
C	T	V	T	H	T	D	L	P	S		
TGC	ACC	GTG	ACC	CAC	ACA	GAC	CTG	CCC	TCG		
P	L	K	Q	T	I	S	R	P	K		
CCA	CTG	AAG	CAG	ACC	ATC	TCC	CGG	CCC	AAG		
G	V	A	L	H	P	D	R	V	Y		
GGG	GTG	GCC	CTG	CAC	AGG	CCC	GAT	GTC	TAC		
L	L	P	P	A	R	E	Q	L	N		
TTG	CTG	CCA	CCA	GCC	CGG	GAG	CAG	CTG	AAC		
L	R	E	S	A	T	I	T	C	L		
CTG	CGG	GAG	TCG	GCC	ACC	ATC	ACG	TGC	CTG		
V	T	G	F	S	P	A	D	V	F		
GTG	ACG	GGC	TTC	TCT	CCC	GCG	GAC	GTC	TTC		
V	Q	W	M	Q	R	G	Q	P	L		
GTG	CAG	TGG	ATG	CAG	AGG	GGG	CAG	CCC	TTG		
S	P	E	K	Y	V	T	S	A	P		
TCC	CCG	GAG	AAG	TAT	GTG	ACC	AGC	GCC	CCA		
M	P	E	P	Q	A	P	G	R	Y		
ATG	CCT	GAG	CCC	CAG	GCC	CCA	GGC	CGG	TAC		
F	A	H	S	I	L	T	V	S	E		
TTC	GCC	CAC	AGC	ATC	CTG	ACC	GTG	TCC	GAA		
E	E	W	N	T	G	E	T	Y	T		
GAG	GAA	TGG	AAC	ACG	GGG	GAG	ACC	TAC	ACC		
C	V	A	H	E	A	L	P	N	R		
TGC	GTG	GCC	CAT	GAG	GCC	CTG	CCC	AAC	AGG		
V	T	E	R	T	V	D	K	S	T		
GTG	ACC	GAG	AGG	ACC	GTG	GAC	AAG	TCC	ACC		
G	K	P	T	L	Y	N	V	S	L		
GGT	AAA	CCC	ACC	CTG	TAC	AAC	GTG	TCC	CTG		
V	M	S	D	T	A	G	T	C	Y		
GTG	ATG	TCC	GAC	ACA	GCT	GGC	ACC	TGC	TAC		
TGA											

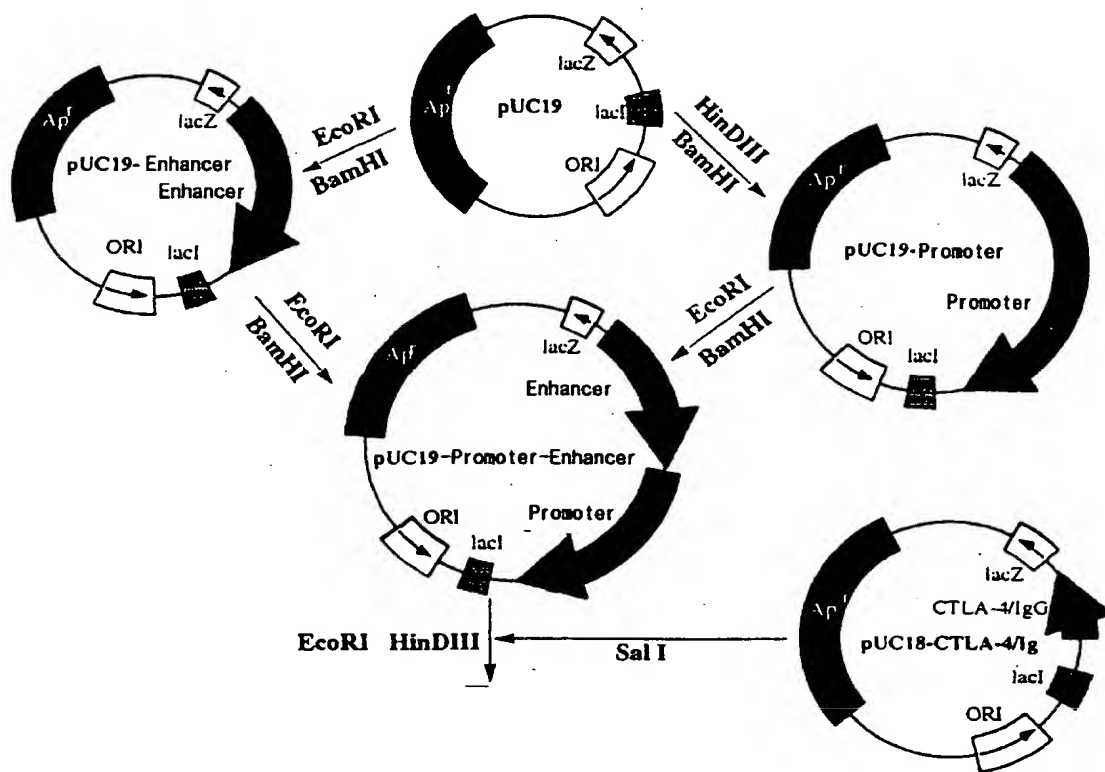
CH₃ region

IgM

CH₄ region

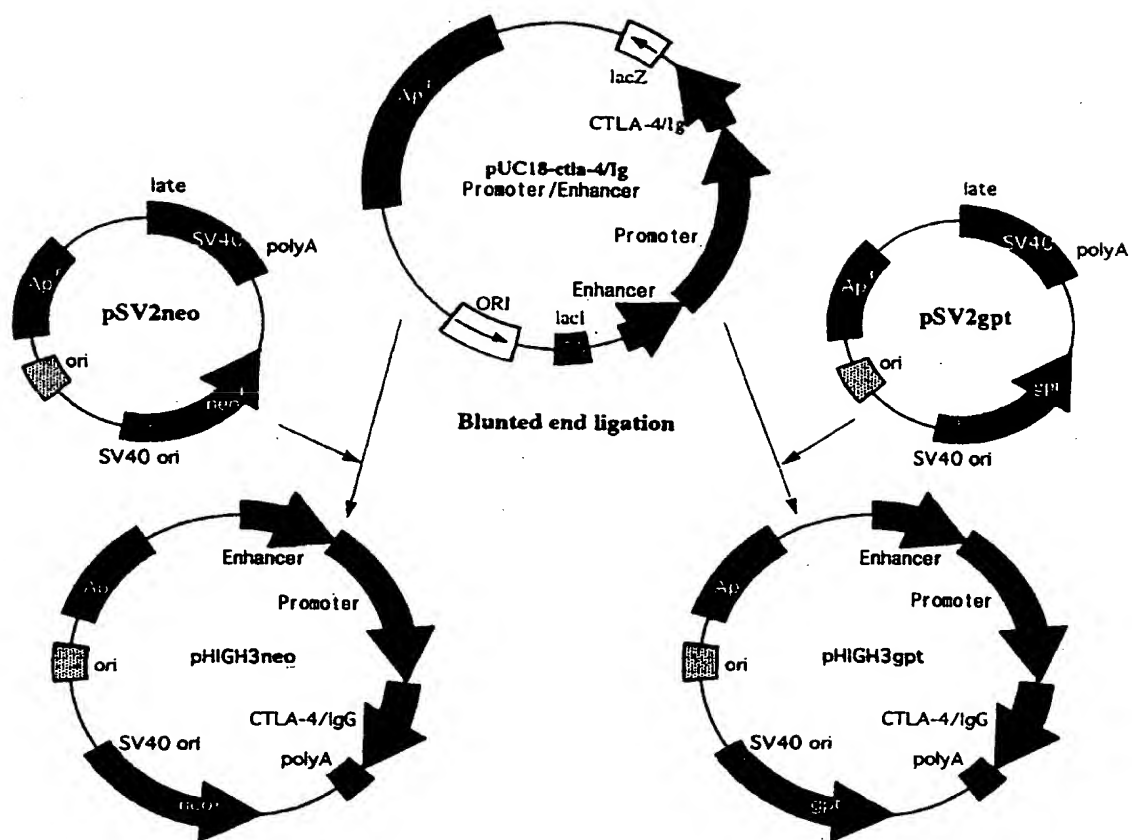
7/12

Fig. 5a



8/12

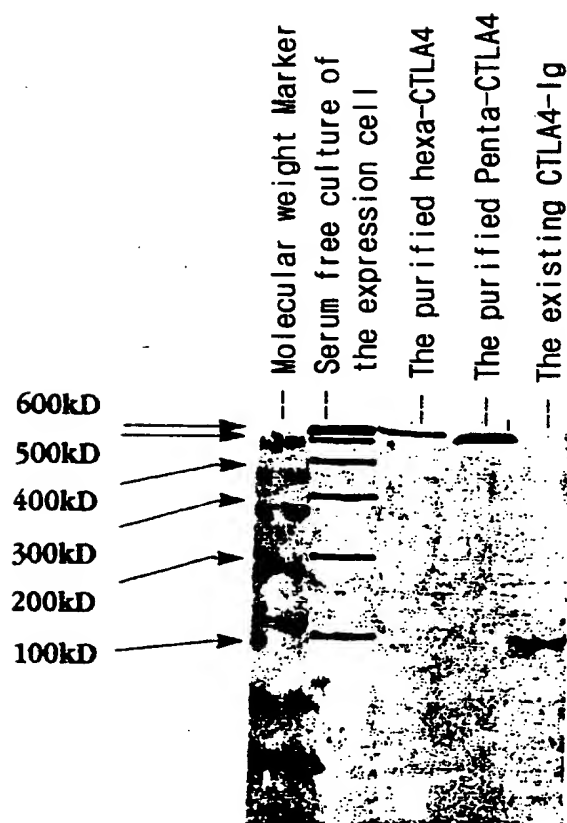
Fig. 5b



9/12

Fig. 6a

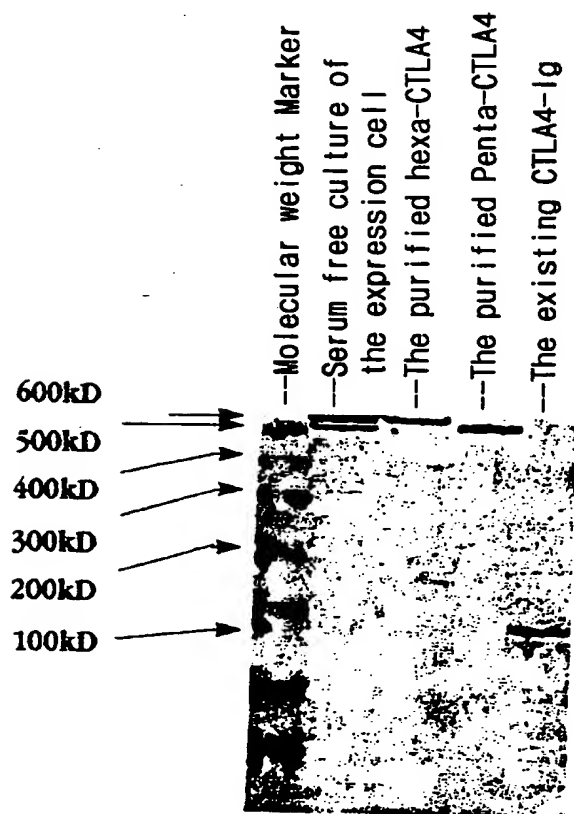
A The properties of the CTLA4-IgG₁-Cys₃₀₈ fusion protein



10/12

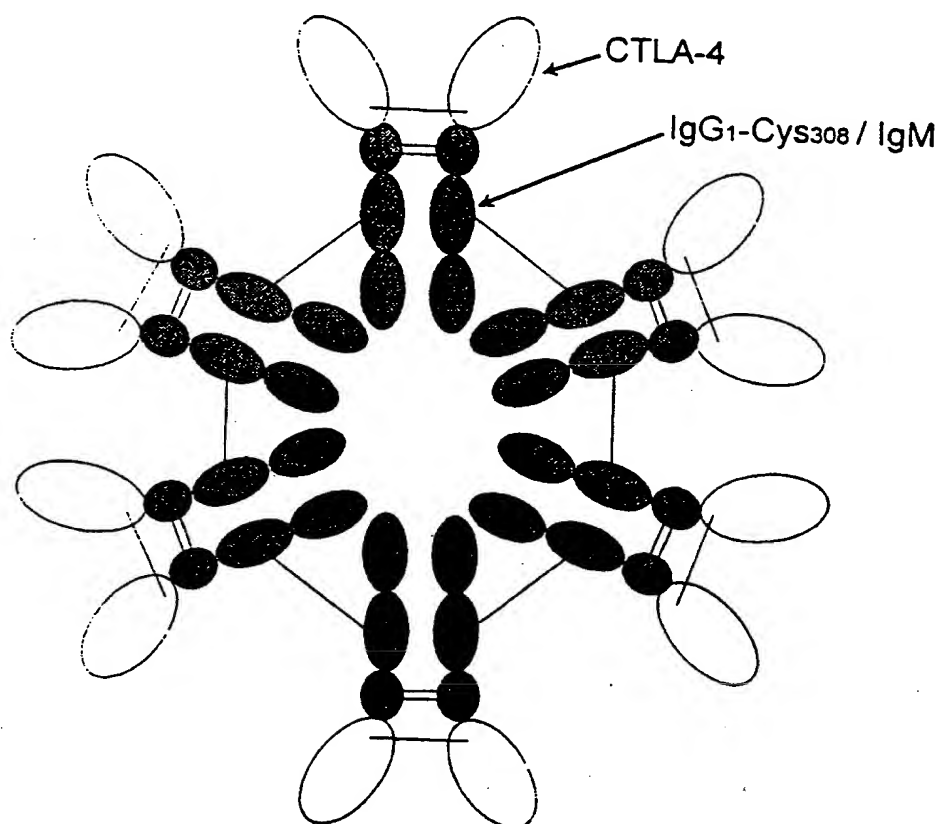
Fig. 6b

B The properties of the CTLA4-IgM fusion protein



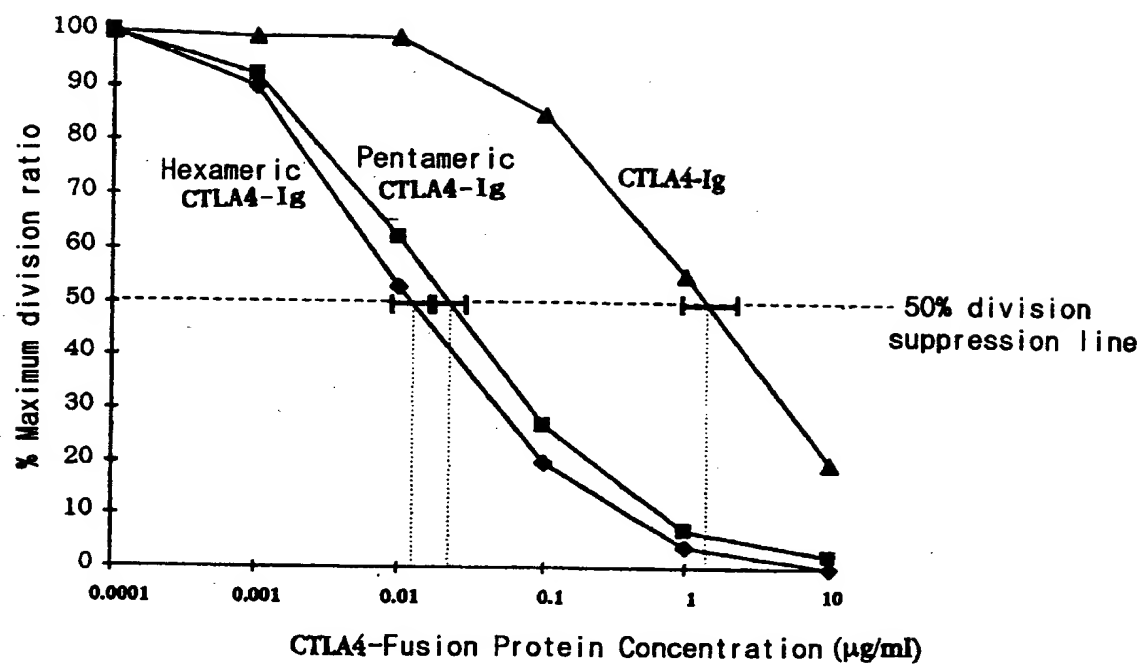
11/12

Fig. 7



12/12

Fig. 8



INTERNATIONAL SEARCH REPORT

International application No.

PCT/KR 98/00009

A. CLASSIFICATION OF SUBJECT MATTER

IPC⁶: C 12 N 15/62; A 61 K 38/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC⁶: C 12 N 15/62; A 61 K 38/00

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WPIL

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	EP 0 682 039 A1 (BRISTOL-MYERS SQUIBB COMPANY) 15 November 1995 (15.11.95), abstract; claims 1,29.	1,6
A	US 5 434 131 A (LINSLEY et al.) 18 July 1995 (18.07.95), abstract; claims 5,6. -----	1,6

☐ Further documents are listed in the continuation of Box C.☒ See patent family annex.

* Special categories of cited documents:

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

04 May 1998 (04.05.98)

Date of mailing of the international search report

15 May 1998 (15.05.98)

Name and mailing address of the ISA/ AT

AUSTRIAN PATENT OFFICE

Kohlmarkt 8-10

A-1014 Vienna

Facsimile No. 1/53424/535

Authorized officer

Wolf

Telephone No. 1/53424/436

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.
PCT/KR 98/00009

In Recherchenbericht angeführtes Patentdokument Patent document cited in search report Document de brevet cité dans le rapport de recherche		Datum der Veröffentlichung Publication date Date de publication	Mitglied(er) der Patentfamilie Patent family member(s) Membre(s) de la famille de brevets	Datum der Veröffentlichung Publication date Date de publication
EP A1	682039	15-11-95	AU A1 16458/95 CA AA 21468/95 FI A0 951801 FI A 951801 IL A0 112343 JP A2 8047391 NO A0 951436 NO A 951436	26-10-95 16-10-95 13-04-96 16-10-95 31-07-95 20-02-96 12-04-96 16-10-95
US A	5434131	18-07-95	AU A1 22400/92 AU B2 661854 CA AA 2110518 EP A1 606217 FI A 935795 FI A0 935795 IL A0 102294 JP T2 65008989 MX A1 9203605 NO A0 934801 NO A 934801 NZ A 243286 NZ A 264712 PT A 100637 WO A1 9300431 ZA A 9204782	25-01-93 10-08-95 07-01-96 20-07-94 22-12-94 22-12-94 14-01-96 13-10-94 01-11-94 23-12-94 21-02-94 26-03-96 26-03-96 31-05-94 07-01-94 27-12-93